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Peroxynitrite formation and apoptosis in transgenic sickle cell mouse kidneys

NORMAN BANK, MILITZA KIROYCHEVA, FAYYAZ AHMED, GILLIAN M. ANTHONY, MARY E. FABRY, RONALD L. NAGEL, and PRAVIN C. SINGHAL

Renal and Hematology Divisions, Department of Medicine, Montefiore Medical Center, and the Albert Einstein College of Medicine and Long Island Jewish Medical Center, Bronx, New York, USA

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Background. In a previous study, nitric oxide synthases (NOS) were found to be strongly expressed in the tubular epithelium of kidneys of a transgenic mouse model of sickle cell disease ($\alpha^{H}\beta^{S}[\beta^{MDD}]$). Because NOS activity is often associated with peroxynitrite formation when superoxide radical ($^{\cdot}O^{-}_{2}$) is present in abundance, we examined the kidneys of sickle cell mice for nitrotyrosine, considered to be a footprint of $ONOO^{-}$.

Methods. Western blot and immunohistochemistry for nitrotyrosine was carried out. Since peroxynitrite and other reactive oxygen radicals are capable of causing apoptosis, we also performed agarose gel electrophoresis of kidney DNA and TUNEL staining of nuclei, indicators of apoptosis.

Results. Nitration of tyrosine residues of three proteins (kD 66, 57 and 22) was found on Western blot of kidney protein extracts of the sickle cell mice. The degree of tyrosine nitration of the 66 kD protein was not significantly different in the control versus transgenic mice, whereas tyrosine nitration of the 57 and 22 kD proteins was clearly increased in transgenic mice. Strong immunostaining for nitrotyrosine was seen in tubular epithelial cells of the sickle cell mice, in close proximity to positive immunostaining of iNOS. Neither iNOS nor nitrotyrosine was expressed in the control mice. DNA "laddering" was found localized to the same zones of the kidney as nitrotyrosine and iNOS immunostaining. TUNEL assay on mouse kidney tissue sections showed minimal tubular cell apoptosis in normal mouse with hypoxia, mild tubular cell apoptosis in sickle cell mouse in room air, and moderate tubular cell apoptosis in sickle cell mouse with hypoxia.

Conclusions. The observations suggest that $ONOO^{-}$ and perhaps other reactive oxygen species are being produced in the sickle cell kidney. The mechanism may be ischemia/reperfusion due to intermittent vascular occlusion by sickle cells. The resulting hypoxia could result in iNOS activation, superoxide radical and peroxynitrite formation. Two consequences of these reactions appear to be nitration of tyrosine residues of some renal proteins and enhanced apoptosis.

Key words: nitric oxide, nitrotyrosine, Western blot, immunohistochemistry, iNOS, DNA strand breaks, hypoxia.

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Several clinical observations in sickle cell patients suggest that nitric oxide (NO) synthesis is increased. These include low blood pressure [1], renal hyperperfusion despite elevated renin/angiotensin blood levels [2–6] and priapism [7, 8], abnormalities that have been associated with an increase in NO production in other clinical conditions. In addition, plasma nitrite, a stable metabolic product of NO, was found to be elevated in patients during sickle cell crisis [9], a finding consistent with augmented production of NO.

Our laboratory previously studied two of the isoenzymes responsible for NO synthesis, the endothelial cell constitutive isoform (ecNOS) and the macrophage inducible isoform (iNOS), in the kidneys of a transgenic mouse model of sickle cell disease ($\alpha^{H}\beta^{S}[\beta^{MDD}]$) [10]. Expression of both were increased [10]. The mechanism of the induction of these enzymes was postulated to be hypoxia secondary to vasoocclusion of small blood vessels within the kidney [10].

It has become evident that under certain experimental conditions, increased NOS activity is associated with production of several reactive oxygen species, such as superoxide radical ($^{\cdot}O_{2}^{-}$), lipid peroxyl radicals (LOO^{-}), hydroxyl radical ($OH^{\cdot}$) and peroxynitrite ($ONOO^{-}$) [11]. Peroxynitrite in particular has been postulated to be responsible for several of the cytotoxic effects previously ascribed to NO under several clinical and experimental conditions [12–15]. For example, in NOS-transfected human renal tubular epithelial cells, eliminating L-arginine from the medium was found to result in $ONOO^{-}$ production, nitrotyrosine formation, and cellular injury [16]. Renal injury caused by cross-clamping of the renal pedicle of rats and mice, followed by reperfusion, was also found to result in renal iNOS and nitrotyrosine expression [17]. Ischemia-reperfusion injury of isolated rat hearts has also been associated with production of $ONOO^{-}$ and nitrotyrosine formation [18].

To evaluate whether increased iNOS expression is associated with formation of peroxynitrite in sickle cell nephropathy, we examined the kidneys of β^{S} transgenic sickle

cell mice for expression of a marker of $ONOO^-$, that is, nitrotyrosine. Since $ONOO^-$ is a potent agent for cell injury, we also carried out DNA electrophoresis and TUNEL staining of cell nuclei in order to assess apoptosis. Strong nitrotyrosine and iNOS immunostaining was found in renal epithelial cells of the medulla of sickle cell mouse kidneys, and nitration of tyrosine residues of several proteins on Western blot. DNA electrophoresis and TUNEL staining showed extensive evidence of apoptosis in the outer and inner medullary regions. The observations indicate that increased activity of iNOS in the kidneys of sickle cell mice is associated with peroxynitrite formation, which may be responsible for accelerated apoptosis.

METHODS

The methods to establish the β^S transgenic line of sickle cell mice used in this study have been described previously [19, 20]. This line was created by the simultaneous microinjection and cointegration of constructs of the locus control regions of the genes responsible for β^S and α hemoglobin chains, on a normal mouse background. The mice with stable expression of α^H and β^S were bred for seven or more generations onto the C57BL/6J background. To achieve higher levels of β^S expression, the transgenic mice were bred with a strain of mice bearing a spontaneous deletion of the mouse β^{major}-globin gene. When the β^{major} deletion was bred to homozygosity, expression of β^S increased to an average of 72.7% [19]. The final mouse model is designated as $\alpha^H\beta^S[\beta^{MDD}]$, indicating the double deletion of mouse β^{major}-globin. The control mice used in this study were of the C57BL/6J strain [20] purchased from Jackson Laboratories (Bar Harbor, ME, USA).

All animals were allowed free access to a mouse pellet diet and water. The mice were between 6 and 10 months of age and weighed between 30 and 40 g at the time of study. They were housed in individual cages under room air conditions or under hypoxic conditions for four to five days in glass environmental chambers (Braintree Scientific, Braintree, MA, USA) filled with constantly flowing 10% O_2/0.5% CO_2/89.5% N_2 gas. Since the β^S transgenic mice manifest low levels of sickling and medullary vasoocclusion under room air conditions, we exposed half of the transgenic mice to chronic hypoxia to increase *in vivo* sickling and renal medulla and papillary vasoocclusion, as previously reported [20, 21]. The other β^S mice were housed in ambient room air in individual metabolic cages. Control mice were also exposed to chronic hypoxia in order to distinguish between the effects of hypoxia *per se* versus vasoocclusion of intrarenal blood vessels, which occurs only in the sickle cell mice [20]. In each of the protocols described below, groups of four mice (2 control, 2 transgenic) were studied simultaneously to minimize variability due to biological supplies.

On the day of study, the mice were anesthetized with ether, the kidneys were rapidly excised through a midline incision and blood was drained from the severed renal artery and vein during washing in ice-cold homogenizing buffer. Kidneys for immunohistochemistry were fixed in 10% buffered formalin. Kidneys for DNA extraction and electrophoresis were frozen immediately in liquid nitrogen. Kidneys for Western blot were prepared immediately.

Sample preparation and protein extraction

Kidneys were homogenized (Ultra Turrax T25; IKA-Labortechnik, Staufen, Germany) in 2 ml of ice-cold homogenizing buffer, (pH 7.5, Tris 50 mM, NaCl 20 mM, EDTA 1 mM, NP-40 0.3%), and 0.1 ml/g kidney wt of a protease inhibitor cocktail consisting of 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64), bestatin, leupeptin, and aprotinin (Sigma Chemical Co., St. Louis, MO, USA). The homogenates were centrifuged at 10,000 rpm at 4°C for 20 minutes. The supernatant was collected and was again centrifuged at 10,000 rpm for two minutes. The protein concentration of the supernatant was determined by the Bradford method (BioRad). Sixty μg of protein was used per well and volume adjusted with SDS loading buffer (62.5 mM Tris-HCl, glycerol 10%, bromphenol blue 1% wt/vol, SDS 2%, mercaptoethanol 2.5%). Samples were then boiled for two to three minutes before loading on 12% SDS separating gel. Electrophoresis was carried out for one hour at 200 volts. Transfer of proteins to nitrocellulose or PVDF membranes was performed at 100 volts for one hour in transfer buffer (25 mM Tris, 192 mM glycine, and 5% methanol).

Western blot

Membranes were blocked in TBS (pH 7.5, 20 mM Tris, 140 mM sodium chloride) containing 3% non-fat milk (TBS-MLK) for 30 minutes with constant agitation, and then were incubated overnight at 4°C with 1 μg/ml of a mouse IgG monoclonal nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA) diluted in freshly prepared TBS-MLK. A positive control was carried out with each experiment, consisting of a range of molecular weight protein standards that had been tyrosine nitrated (supplied by Upstate Biotechnology). Negative control was carried out by preincubating the primary antibody with 10 mM nitrotyrosine (30 min, 20°C; Sigma) and adjusting the pH to 7.2. Membranes were washed twice with water and incubated with goat or sheep anti-mouse IgG linked to horseradish peroxidase (1:2000 dilution in TBS-MLK; Amersham) for 1.5 hours at room temperature. Membranes were washed first with water and then TBS-0.05% Tween for five minutes. They were then rinsed five times in water. ECL (Amersham) was used as the visualization method.

Immunohistochemistry

Kidney tissue sections from control and β^S transgenic mice were processed simultaneously for immunostaining of

nitrotyrosine and iNOS. Simultaneous processing, using identical reagents and timing, permits semiquantitative comparisons of staining intensity. Four to 6 micron-thick sections of formalin-fixed kidneys were cut, dewaxed twice in xylene, rehydrated in ethanol, and washed. Adjacent sections from each kidney were examined with affinity purified polyclonal antibodies directed against mouse macrophage inducible nitric oxide synthase (iNOS) or a rabbit polyclonal IgG antibody directed against nitrotyrosine.

Nitrotyrosine. Formalin-fixed sections were placed in 0.03% H_2O_2 in methanol for 30 minutes to neutralize endogenous peroxidases and were blocked with normal goat serum containing 0.5% BSA for 30 minutes. They were then washed in PBS for 15 minutes, and the primary antibody (1:100 dilution of immunoaffinity purified rabbit polyclonal IgG directed against nitrotyrosine; Upstate Biotechnology, Lake Placid, NY, USA) was added and incubated at 4°C overnight in a humidified chamber. The secondary antibody was 1% goat biotinylated anti-rabbit IgG-biotin, visualized by reaction with avidin-biotin peroxidase (Vectastain Elite ABC kit, Vector Labs, Burlingame, CA, USA). The sections were then incubated with fresh diaminobenzidine tetrahydrochloride (DAB) solution (Sigma) for seven minutes at room temperature. Positive controls were carried out on kidney sections from normal control mice by incubation with a peroxynitrite solution, following instructions of Upstate Biotechnology. After incubation, the primary and secondary antibodies were added as above. Negative controls were performed by exposure of slides from transgenic mouse kidneys to 10 mM nitrotyrosine in PBS, which blocks binding of the first antibody. Reagents for the positive and negative controls were obtained from Upstate Biotechnology. Sections were counterstained with hematoxylin for 30 to 45 seconds.

iNOS. Kidney slices adjacent to those that had been used for nitrotyrosine were examined for iNOS immunostaining. The slides were heated at 60°C for 30 minutes, washed in xylene, 100% ethanol, 95% ethanol and fixed in 4% formalin for 30 minutes at room temperature. Nonspecific binding was blocked with normal goat serum containing 0.5% BSA. The slides were then incubated with an affinity-purified mouse macrophage polyclonal iNOS antibody raised in rabbits (Transduction Laboratories, Lexington, KY, USA), 16 μg/ml in PBS at 4°C overnight in a humidified chamber. The secondary antibody (biotinylated anti-rabbit antibody) was added and incubated for one hour at room temperature. An alkaline phosphatase substrate was then applied (Vectastain ABC-AP reagent and Vector red substrate kit; Vector Laboratories) and incubated in the dark for 25 minutes. The resulting diazo compound is intensely red. The slides were counterstained with hematoxylin for 45 seconds at room temperature. Procedures recommended by Vector were closely followed.

Slides from each kidney, incubated with either nitrotyrosine or iNOS antibody, were examined consecutively. Closely comparable areas of each kidney, stained with one of the two primary antibodies, were photographed at ×100 magnification, using a Nikon HFX-DX microscope equipped with an automated Nikon camera system, NFX-35. A blue filter was used to accentuate the contrast between positive staining and background staining and to select areas for photography.

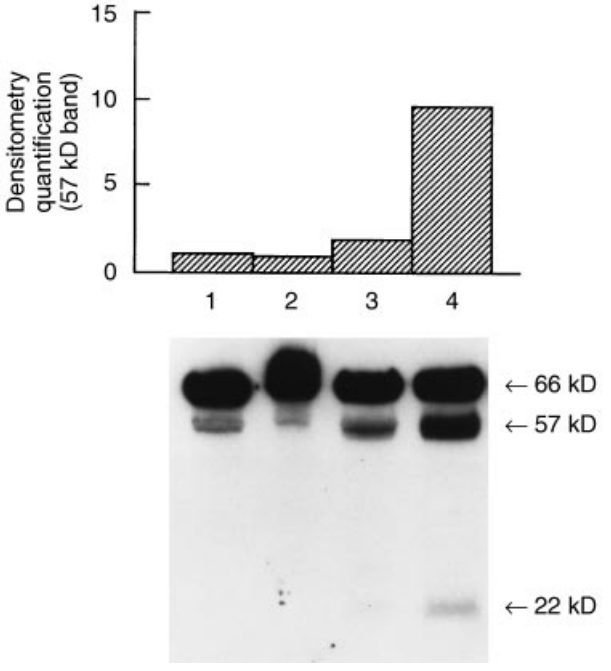

Fig. 1. *Top:* **Densitometric quantification of tyrosine nitration of the 57 kD protein expressed as the ratio of the means compared to normal mice from 7 groups of 4 mice each.** *Bottom:* Western blot of renal protein extracts using a monoclonal antibody directed against nitrotyrosine (exposure 1 min). Lane 1, normal mouse; lane 2, hypoxic normal mouse; lane 3, sickle cell mouse; lane 4, hypoxic sickle cell mouse.

Renal cortical DNA isolation and gel electrophoresis

The renal cortex of kidneys was dissected away from the rest of the kidney, and the cortical tissue was lysed in DNA lysis buffer (1% Nondet P-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5, 10 μl per 10^6 cells, minimum 50 μl). Lysates were centrifuged for five minutes, 1600 × g, supernatants were collected and extractions were repeated. The supernatants were brought to 1% SDS, treated with RNAse for two hours at 56°C and digested with proteinase K for two hours at 37°C. Ammonium acetate (10 mM), 1/2 volume was added and DNA precipitated with 2.5 volumes of 100% ETOH. Pellet was dissolved in TE buffer and separated by electrophoresis in 1.6% agarose gel at room temperature.

TUNEL assay

We used the ENZO ApopDETEK cell death assay system to locate the occurrence of renal cell apoptosis in tissue sections. This system provides a very high quality terminal deoxynucleotide transferase to incorporate Bio-16-dUTP onto the 3′-OH termini in the DNA of the

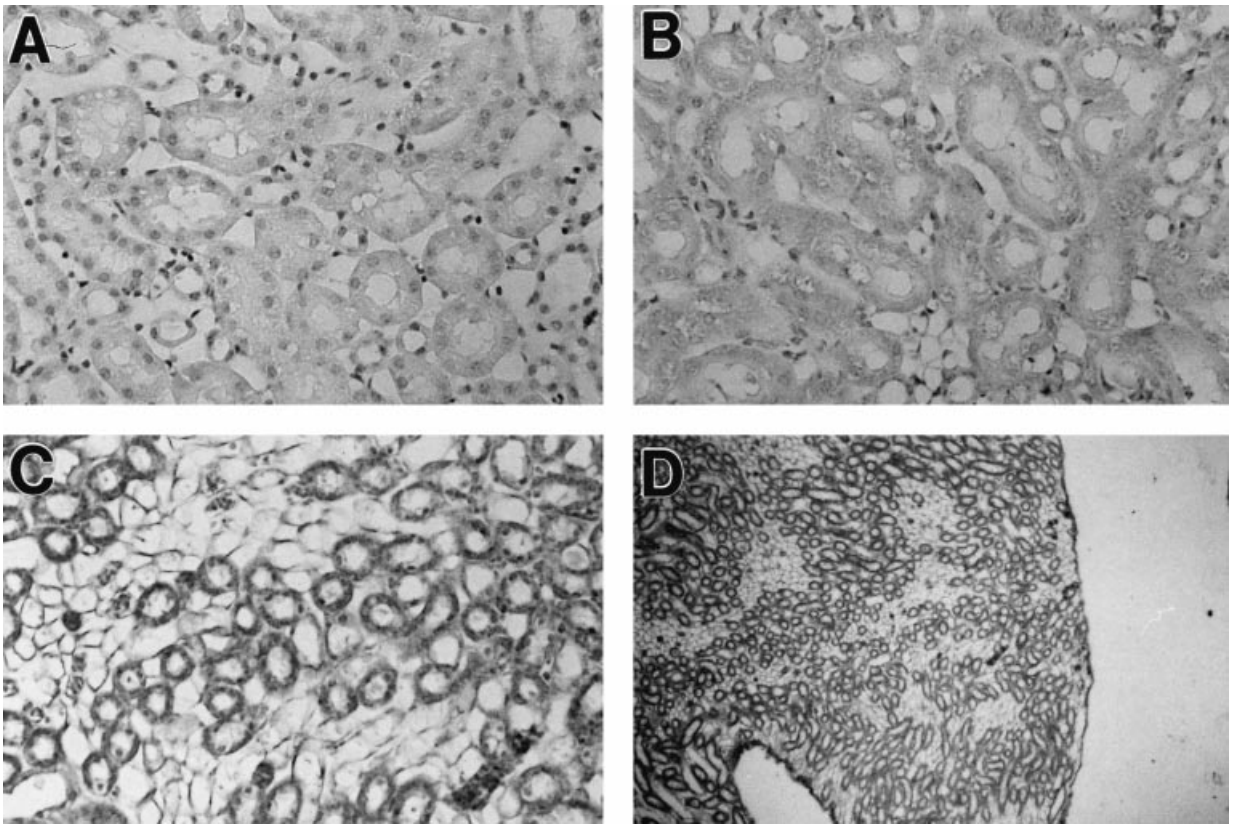

Fig. 2. Immunohistochemistry staining of nitrotyrosine in mouse renal medulla and papilla. (*A*) Normal mouse. (*B*) β^S sickle cell mouse, negative control (Methods section). (*C*) β^S sickle cell mouse renal medulla. (*D*) β^S sickle cell mouse renal papilla.

specimen. The incorporated Bio-16-dUTP is then detected with horseradish peroxidase-aminoethylcarbazole *in situ* detection system. In brief, paraffin tissue sections from control and experimental mice were deparaffinized and permeabilized with proteinase K. Endogenous peroxidase activity was inactivated by treating the tissue sections with 0.5 ml of Quench Reagent (ENZO Diagnostics, Inc., Farmingdale, NY, USA). Sections were incubated with equilibration buffer followed by a reaction mixture (terminal transferase) for 30 minutes. Slides were washed with wash buffer and treated with reagents supplied in the ENZO horseradish peroxidase-aminoethylcarbazole *in situ* detection kit. Slides were examined under a light microscope and graded for the occurrence of apoptosis.

RESULTS

Western blots

Figure 1 (bottom) shows a Western blot of renal protein extracts of mouse kidneys, using a monoclonal antibody directed against nitrotyrosine. Three different molecular weight proteins were detected by the antibody, corresponding approximately to 66, 57 and 22 kD. Tyrosine nitration of the 66 kD band was not significantly different in the normal versus the transgenic mice. However, tyrosine nitration of both the 57 and 22 kD proteins was clearly increased in the transgenic mice, especially when exposed to 10% O_2 for five days. The densitometric quantification of nitrotyrosine of the 57 kD protein, shown in the upper portion of Figure 1, represents the analysis of seven groups of four mice each. Nitration of the 57 kD protein was increased on average by 1.8-fold in transgenic mice versus control mice maintained in room air ($P < 0.01$), and by 9.2-fold in the transgenic mice exposed to hypoxic conditions ($P < 0.001$). Nitration of the 22 kD protein was observed only in some immunoblots of transgenic mice housed in room air, but was observed consistently in the transgenics exposed to hypoxic conditions. No nitration of the 22 kD protein was seen in normal control mice. Preincubation of anti-nitrotyrosine antibody with excess 3-nitrotyrosine (10 mM) completely blocked the immunoreactivity of the proteins (results not shown). Coomassie blue stained SDS gels confirmed that similar amounts of proteins were loaded in each lane.

Immunohistochemistry

Sections of normal and transgenic sickle cell mouse kidneys stained for nitrotyrosine are shown in Figure 2. No staining was found in any of the four control C57BL/6J mice (Fig. 2A), whereas strongly positive staining was evident in all eight sickle cell mice studied. Nitrotyrosine was found in tubular epithelial cells in both the cortex and medulla, but strongest staining was seen in the inner medulla and papilla (Fig. 2 C, D). Figure 2B shows a

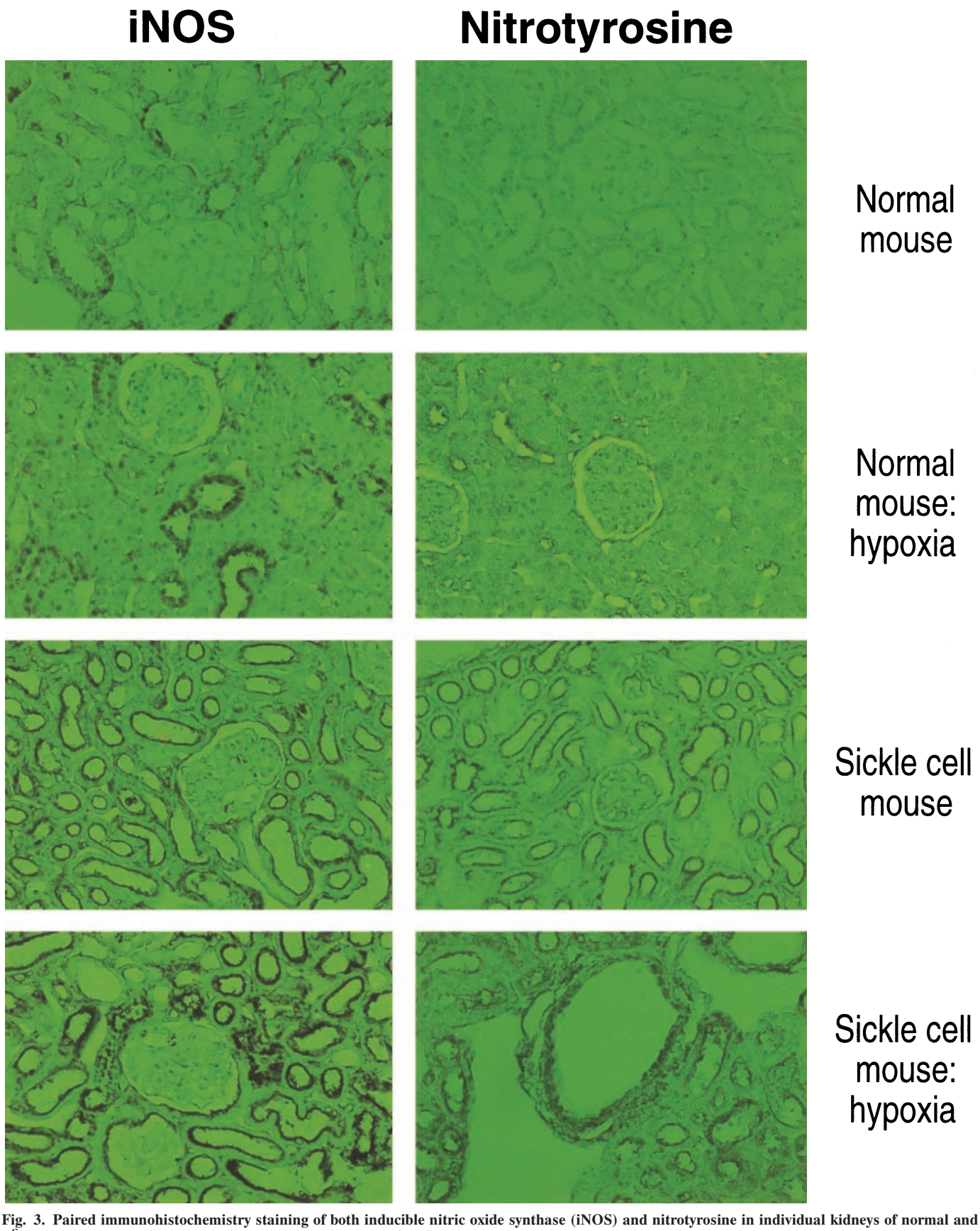

Fig. 3. Paired immunohistochemistry staining of both inducible nitric oxide synthase (iNOS) and nitrotyrosine in individual kidneys of normal and β^S sickle cell mice. Paired photomicrographs are from closely adjacent areas.

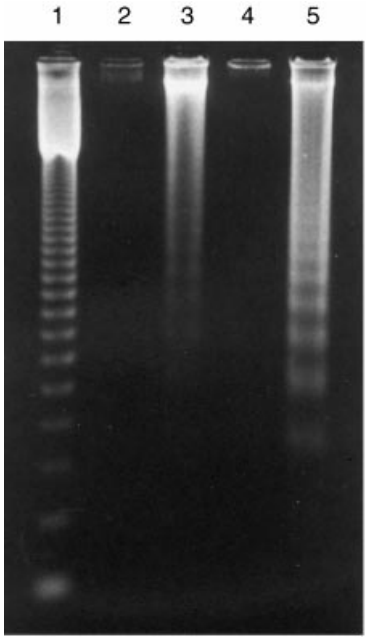

Fig. 4. Agarose gel electrophoresis of DNA extracted from renal cells. Lane 1, 123 kb marker; lane 2, normal mouse; lane 3, sickle cell mouse; lane 4, hypoxic normal mouse; lane 5, hypoxic sickle cell mouse.

negative control carried out on a section of a sickle cell mouse kidney in which the slide was incubated with 3-nitrotyrosine before addition of the primary antibody. This clearly blocked reaction with the primary antibody. Positive controls consisted of kidney sections of normal mice incubated with peroxynitrite prior to carrying out immunohistochemistry. This was found to result in positive immunostaining of tubular epithelial cells (not shown).

Figure 3 compares the immunostaining of nitrotyrosine with iNOS in closely adjacent paired kidney sections of four mice. The results are representative of three additional groups of four mice each. Under room air conditions, no staining for either nitrotyrosine or iNOS was found in the normal mice. Exposure of normal mice to hypoxia for four to five days resulted in scattered iNOS staining in a few distal nephron segments in the cortex and medulla, but no nitrotyrosine staining was seen. In contrast, in the sickle cell mice, strongly positive immunoreactivity was observed for both iNOS and nitrotyrosine under both room air and chronic hypoxic conditions. The nitrotyrosine staining was strongest in the luminal membrane, but was also expressed in the basolateral membrane. The glomeruli were negative. Positive staining for nitrotyrosine was also found in the walls of blood vessels, as shown in Figure 3. The iNOS immunostaining in sickle cell mouse kidneys is similar to that reported previously by our laboratory [10].

DNA electrophoresis

Figure 4 shows an agarose gel electrophoresis of DNA extracted from cortical tissue. As can be seen, there was no strand breakage of DNA in control mice housed under either room air or hypoxic conditions. In sharp contrast, multiple nucleoendosome bands were seen in the gels of β^S mouse kidneys, indicating extensive double strand breakage.

TUNEL assay for apoptosis

Figure 5 shows representative photomicrographs of TUNEL-positive apoptotic cells in the sections of renal medulla of sickle cell mice maintained under room air conditions as well as hypoxic conditions. It is evident that uptake of Bio-16-dUTP occurred in the nuclei of β^S mouse epithelial cells, the number of effected cells being greater in the hypoxic β^s mouse. Most of the positive nuclei were in the medullary collecting ducts, but were also seen in the cortex in smaller numbers. As can be seen in Figure 5A, no evidence of DNA nicking was evident in the control mouse, but low grade evidence of apoptosis was present in the hypoxic control mouse (Fig. 5B).

DISCUSSION

The major end product of the reaction between L-arginine and nitric oxide synthases is nitric oxide (NO). NO synthesis depends on an adequate supply of the precursor, L-arginine, and several co-factors including molecular oxygen, NADPH and tetrahydrobiopterin [22–26]. Under experimental conditions of severe L-arginine depletion [16, 27, 28], or ischemia/reperfusion of the heart, lung or kidney [17, 18, 29], there is induction of NOS, increased superoxide radical production, and formation of the potent oxidizing agent, peroxynitrite. Tissue injury that occurs under these experimental conditions has been attributed by a number of investigators to peroxynitrite, since immunostaining or increased immunoblot evidence of nitrotyrosine was found in these studies [reviewed in 30]. Nitrotyrosine formation results from the specific reaction between peroxynitrite ($ONOO^-$) and tyrosine residues of various proteins [30, 31], and therefore nitrotyrosine serves as presumptive evidence of $ONOO^-$ formation.

In the present study, we examined the kidneys of a transgenic mouse model of sickle cell disease ($\alpha^H\beta^S[\beta^{MDD}]$) for evidence of nitrotyrosine expression, by Western blot and immunohistochemistry. As shown in Figure 1, nitrotyrosine reaction was found in two protein bands in normal mice whereas three distinct bands were seen in the transgenic mouse kidney extracts. The presence of nitrated proteins in normal conditions is not surprising as it has been reported previously [32], and may possibly derive from low levels of NO and O^-_2 produced constitutively. The level of tyrosine nitration was quantitatively greater in kidney protein extracts from sickle cell mice, and exposure of these mice to hypoxia led to a further marked increase (Fig. 1). Exposure of control mice to hypoxia did not consistently alter tyrosine nitration. Since the mouse IgG monoclonal antibody detects the quantity of nitrated tyrosine residues, the increased reaction seen in the sickle

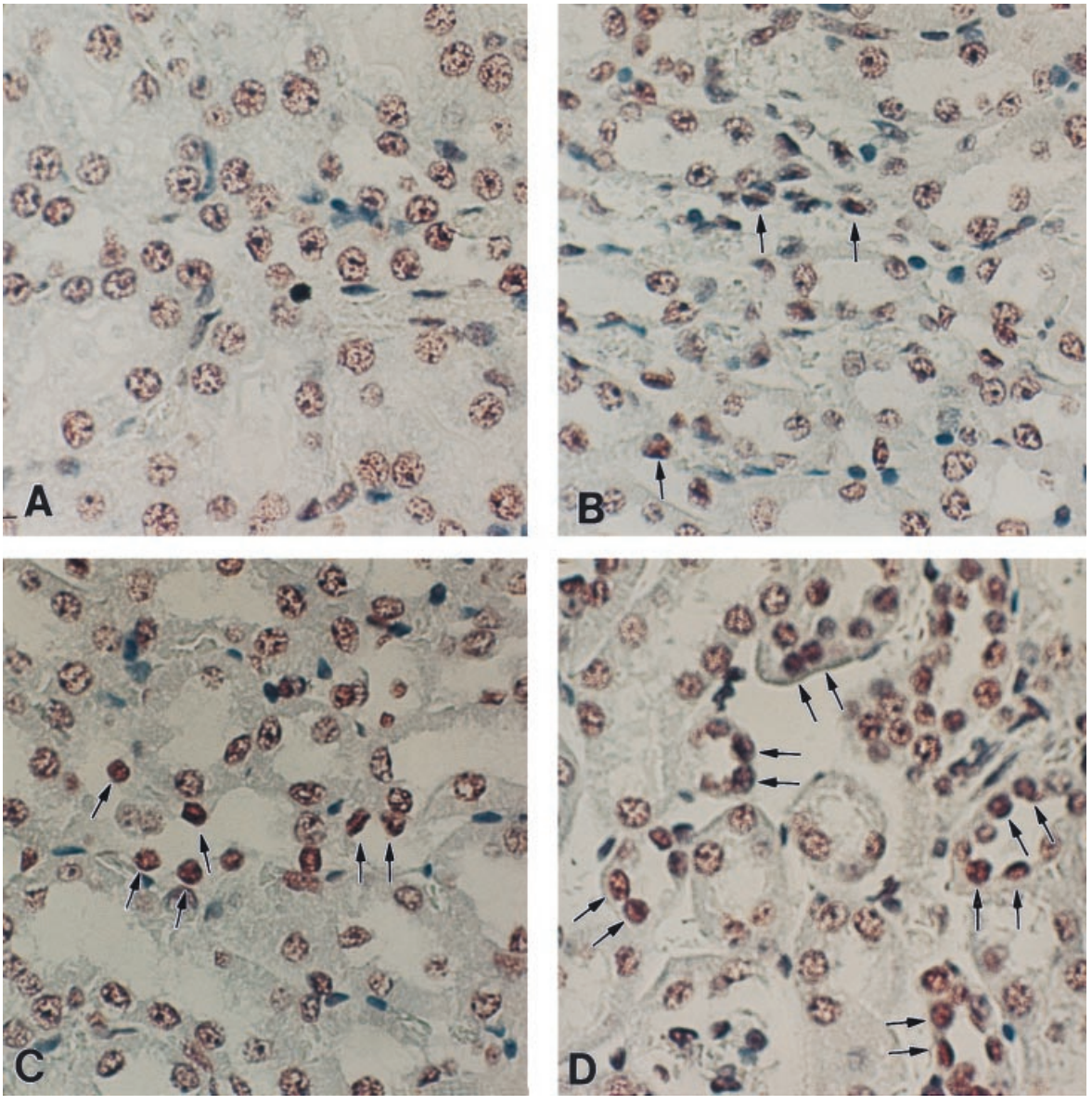

Fig. 5. Occurrence of apoptosis in mouse renal medulla detected by TUNEL method. TUNEL-positive cells are indicated by arrows. (*A*) Normal mouse. (*B*) Hypoxic normal mouse. (*C*) Sickle cell mouse. (*D*) Hypoxic sickle cell mouse.

cell mice could be due to either increased quantities of protein in the transgenic mice or to increased nitration of similar protein quantities. The Western blots do not distinguish between these two possibilities. However, there were no differences in protein quantities loaded onto each lane when examined in Coomassie blue-stained SDS gels (not shown). Hence, the increased expression of nitrotyrosine on Western blot presumably reflects increased nitration of tyrosine residues of equal quantities of protein.

Representative immunohistochemistry results, shown in Figures 2 and 3, demonstrate that nitrotyrosine staining was strongly positive in the β^S mice but was minimal or absent in the normal mice. Positive staining was observed in cortical distal nephron segments, the outer and inner medulla, and the papilla of the transgenic mice, whereas in normal control mice, no immunostaining for nitrotyrosine was found under either room air or hypoxic conditions. These observations provide strong presumptive evidence for formation of peroxynitrite in the tubular epithelial cells of sickle cell mice, and confirm the findings of nitrotyrosine on Western blot. In addition, paired staining for iNOS showed that the zones of the kidney where nitrotyrosine staining was found were in close proximity to those where iNOS was seen (Fig. 3). Staining for both was found in cortical collecting tubules, and collecting ducts of the outer and inner medulla and papilla. The photomicrographs in Figure 3 show iNOS and nitrotyrosine reactions in the same kidney of each of the four mice, focused on closely comparable regions, although they do not represent precise co-localization. These observations nevertheless suggest

that there may be site-specific localized production of NO and $ONOO^-$ that leads to nitrotyrosine formation in closely adjacent regions. Consistent with this view is the recent report of Bosse and Bachmann, who found evidence of co-localization of nitrotyrosine and NOS activity in kidneys of rats with unilateral renal artery stenosis [33].

A number of studies have shown that peroxynitrite can mediate DNA double strand breakage, an expression of apoptosis [34–38]. In the kidney, histologic evidence of apoptosis and/or DNA "laddering" has been demonstrated in proximal tubular epithelial cells [39] and mesangial cells [40] under several different experimental conditions, such as exposure to H_2O_2 [39, 40], ischemia/reperfusion injury [41, 42] and transplant rejection [43]. The common denominator in these conditions is thought to be enhanced production of reactive oxygen species, such as $^{\bullet}O_2^-$, H_2O_2, $OH^{\bullet}$ and $ONOO^-$ in excess of endogenous antioxidant protection [30, 34, 44, 45]. As shown in Figure 4, DNA strand breakage was clearly present in the sickle cell mouse kidneys and apoptosis by TUNEL assay.

The mechanism of $ONOO^-$ and nitrotyrosine formation in the sickle cell mice is not yet clear. In isolated NOS-transfected human kidney epithelial cells, depletion of L-arginine resulted in a fall in NO and a rise in $^{\bullet}O_2^-$ production, accompanied by peroxynitrite formation and evidence of cell toxicity [16]. A similar sequence of events was found in murine macrophages depleted of cytosolic L-arginine [28]. Although L-arginine depletion has not been demonstrated in whole tissues or organs, it is conceivable that this may occur in some pathological conditions. The source of L-arginine in the kidney is most likely that which is synthesized in renal tubular cells from citrulline and aspartate, utilizing ATP [46]. The quantity of arginine normally used in NO production by the constitutive isoforms of NOS is very small as compared with that utilized in intermediary metabolic reactions [46]. Therefore, it is very unlikely that arginine deficiency would develop under physiological conditions. Moreover, citrulline, an end-product of NO production, can be re-cycled for regenerating arginine. However, when iNOS is induced and the high output pathway of NO synthesis is activated, L-arginine concentrations may become rate-limiting, favoring synthesis of $^{\bullet}O_2^-$ rather than NO. (The K_m for the L-arginine reaction with iNOS is 2.8 μM [47].) ATP is required for L-arginine synthesis [46], and ischemia/reperfusion injury due to vasoocclusion might deplete ATP levels. In addition, recent studies have shown that experimental conditions that induce iNOS, including hypoxia, can also increase arginase activity [48, 49] in some tissues. Increased arginase activity could contribute to lowering cytosolic level of L-arginine. Alternatively to this hypothesis, a rise in tissue $^{\bullet}O_2^-$ may occur due to reduced endogenous dismutases or to increased production via other chemical reactions, thereby providing the necessary concentrations of $^{\bullet}O_2^-$ for $ONOO^-$ formation.

The finding of apoptosis and nitrotyrosine formation in the transgenic sickle cell kidney may be causally related to some of the functional and histologic pathology in this disease. Ischemia/reperfusion-mediated apoptosis could result in tubulointerstitial changes, loss of functioning nephrons, and eventual scarring [50, 51]. Thus, structural damage in sickle cell kidneys may be the consequence of hypoxia-induced iNOS activity and superoxide formation, leading to peroxynitrite formation and accelerated apoptosis.

The functional consequences of nitration of the renal protein tyrosine residues found on Western blot are presently unknown. In other systems, nitration of tyrosine residues by peroxynitrite has been found to inactivate prostacyclin synthase [52], to interfere with tyrosine phosphorylation signal transduction [53, 54], to inhibit epidermal growth factor receptor tyrosine kinase [55], and to inactivate aconitase [56]. Whether these and/or other functional disturbances result from tyrosine nitration in the sickle cell kidney will require further study.

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Reprint requests to Norman Bank, M.D., Renal Division, Montefiore Medical Center, 111 East 210 Street, Bronx, New York 10467, USA.

APPENDIX

Abbreviations used in this paper are: AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; ATP, adenosine 5′-triphosphate; DAB, diaminobenzidine tetrahydrochloride; ecNOS, endothelial cell constitutive isoform of nitric oxide synthesis; iNOS, inducible isoform of nitric oxide synthase; LOO^-, lipid peroxyl radicals; NO, nitric oxide; $^{\bullet}O_2^-$, superoxide radical; $OH^{\bullet}$, hydroxyl radical; $ONOO^-$, peroxynitrite; TUNEL, terminal deoxytransferase uridine triphosphate nick end-labeling.

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